

EXHIBIT G



A non-invasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study

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Summary

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Background Use of free fetal DNA to diagnose fetal chromosomal abnormalities has been hindered by the inability to distinguish fetal DNA from maternal DNA. Our aim was to establish whether single nucleotide polymorphisms (SNPs) can be used to distinguish fetal DNA from maternal DNA—and to determine the number of fetal chromosomes—in maternal blood samples.

Methods Formaldehyde-treated blood samples from 60 pregnant women and the stated biological fathers were analysed. Maternal plasma fractions were quantified at multiple SNPs, and the ratio of the unique fetal allele signal to the combined maternal and fetal allele signal calculated. The mean ratios of SNPs on chromosomes 13 and 21 were compared to test for potential fetal chromosomal abnormalities.

Findings The mean proportion of free fetal DNA was 34·0% (median 32·5%, range 17·0–93·8). We identified three samples with significant differences in the fetal DNA ratios for chromosome 13 and chromosome 21, indicative of trisomy 21; the remaining 57 samples were deemed to be normal. Amniocentesis or newborn reports from the clinical sites confirmed that the copy number of fetal chromosomes 13 and 21 was established correctly for 58 of the 60 samples, identifying 56 of the 57 normal samples, and two of the three trisomy 21 samples. Of the incorrectly identified samples, one was a false negative and one was a false positive. The sensitivity and positive predictive value were both 66·7% (95% CI 12·5–98·2) and the specificity and negative predictive values were both 98·2% (89·4–99·9).

Interpretation The copy number of chromosomes of interest can be directly established from maternal plasma. Such a non-invasive prenatal test could provide a useful complement to currently used screening tests.

Introduction

Available protocols for prenatal diagnosis of aneuploidy are limited by several factors. Screening tests—eg, nuchal translucency and the quadruple screen—are non-invasive, but diagnosis requires further invasive testing.¹ Invasive diagnostic tests—eg, amniocentesis and chorionic villus sampling—are about 99% accurate in identifying the spectrum of chromosomal abnormalities, but are associated with increased risks to the pregnancy.^{2–7} Development of non-invasive tests that yield diagnostic results would be a useful advancement in prenatal care.

Analysis of fetal cells and free fetal DNA in the maternal circulation provides an alternative to existing prenatal tests.^{8–14} The use of free fetal DNA has been reported in the diagnosis of achondroplasia and myotonic dystrophy, to determine fetal sex,^{15–17} and in fetal rhesus D genotyping.¹⁸ However, two major issues have restricted the clinical use of analysis of free fetal DNA. First, little free fetal DNA exists in the maternal circulation, with initial studies reporting a mean of only 3·4% free fetal DNA in the late first trimester to mid second trimester.¹⁹ Second, in a heterogeneous mixture of maternal and fetal DNA it is difficult to distinguish fetal chromosomes of clinical interest—eg, chromosomes 13, 18, and 21—from maternal chromosomes.

We have previously reported that careful sample processing and the addition of formaldehyde increased

the proportion of free fetal DNA recovered from the maternal circulation to about 25%.²⁰ Having increased the proportion of free fetal DNA, one major challenge remained: how to determine the copy number of fetal chromosomes in a heterogeneous mixture of maternal and fetal DNA.

Sequencing of the human genome has led to the discovery of variation in base sequences in individuals, referred to as single nucleotide polymorphisms (SNPs). The use of SNPs for the detection of trisomy 21 has been described from amniotic fluid specimens.²¹ Amniotic fluid, however, contains a 100% sample of fetal DNA compared with the heterogeneous mixture of maternal and fetal DNA seen in maternal plasma. We postulated that an approach that used multiple SNPs, and quantification of an allele ratio for these SNPs in a maternal blood sample, could indicate the presence or absence of fetal aneuploidy. This technique would require neither cell separation nor isolation of free fetal DNA.

At certain SNP sites, the maternal genome will be homozygous for a nucleotide—eg, guanine (G/G)—while at the same site, the paternal genome might be homozygous for a different nucleotide—eg, thymine (T/T; figure 1). Since one copy of each chromosome is inherited from each parent, the fetal genome will be heterozygous (G/T) at the SNP site (figure 1). In the plasma DNA of the maternal blood sample, the presence of thymine at the SNP site represents a unique fetal

signal in the maternal DNA background (figure 1). Our aim was to establish a SNP ratio to identify fetal trisomy 21 from maternal plasma and to develop an approach to quantify and compare allele ratios for SNP sites on chromosomes 13 and 21.

Methods

Procedures

A network of ten clinical sites was established to gather specimens. Each site was involved in perinatal diagnosis of the patients involved. Clinical sites received institutional review board approval before patient enrolment. Patients and the stated biological fathers were aged 18 years or older; only patients with singleton pregnancies were included. Written informed consent was obtained before participation. All test results were compared with amniocentesis or newborn reports obtained from the clinical sites. The study was done between January, 2004, and August, 2006, during which time the technology was developed and refined, and 60 samples were gathered and processed sequentially.

Blood samples were taken from the pregnant women and also from the stated biological fathers. About 35 mL of blood was gathered from all patients (range 25–50 mL). 0.225 mL of a 10% neutral buffered solution containing formaldehyde (4% w/v) was added to all tubes of maternal blood immediately after the blood was drawn. Specimens were picked up the same day at regional clinical sites or shipped by commercial carrier for overnight delivery. Blood was stored at 4°C until processed. The samples were shipped to our central laboratory for analysis. Laboratory personnel were masked as to the identity of the samples with a numerical coding system.

Plasma and buffy coat samples were isolated in accordance with methods described previously.²⁰ Genomic DNA was purified from both the plasma fraction and buffy coat of the same maternal blood sample, and the buffy coat fraction of the paternal sample, with the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). DNA samples were eluted in 2 mL of DNase/RNase-free water. Plasma DNA was concentrated to 50–70 µL with a 10 kDa nominal molecular weight cutoff filter (Millipore, Bedford, MA, USA). The concentrated plasma DNA was split equally into three replicates that were processed separately for detection of fetal signals. 10 µL of each replicate was amplified with the GenomePlex Whole Genome Amplification Kit (Sigma-Aldrich USA, St Louis, MO, USA).

Before analysis of the plasma DNA, maternal and paternal buffy coat samples were analysed to identify two categories of SNPs. In category one, the maternal and paternal samples were homozygous for different alleles at the SNP site. Therefore, the fetal DNA from the plasma was expected to be heterozygous at these sites. In category two, the maternal sample was homozygous, and the paternal sample was heterozygous at the same SNP site. Therefore, there was a 50% chance that the allele

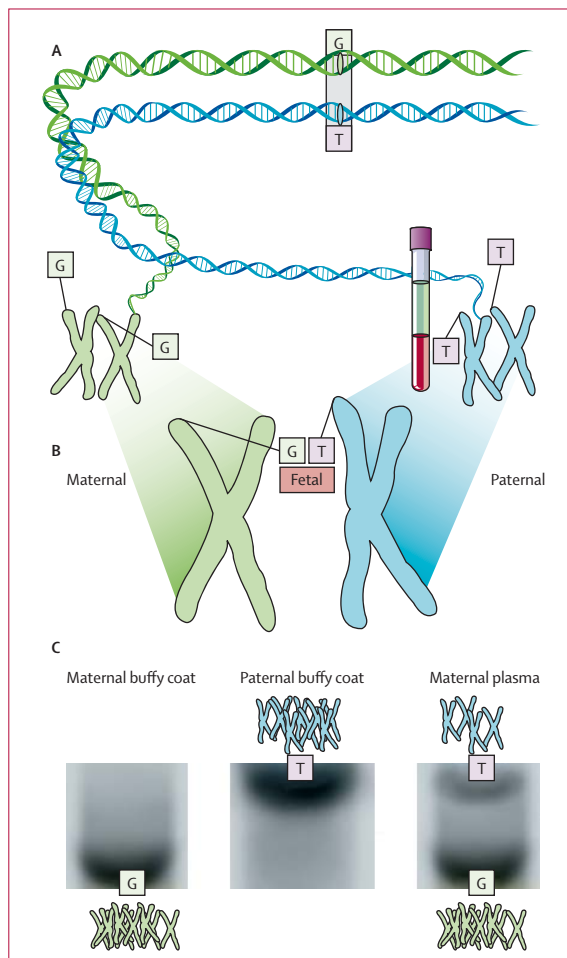


Figure 1: Inheritance of single nucleotide polymorphisms

(A) Single nucleotide polymorphisms (SNPs) are the greatest single source of natural variation in the human genome and can be used to determine the copy number of fetal chromosomes. (B) At certain sites within the fetal genome, the inherited paternal allele ("T") will differ from the inherited maternal allele ("G"). (C) Presence of the paternal allele in the plasma provides a genetic beacon to distinguish fetal DNA from maternal DNA.

inherited by the fetus would be different from the maternal allele. Only SNPs showing a unique fetal allele in the maternal plasma were quantified.

SNPs were chosen so that their sequences fit the following criteria: the nucleotide at ± 2 bp from the SNP site matched one of the two alleles located at the SNP site, whereas the nucleotide at ± 1 bp from the same site contained neither of the two alleles located at the SNP site.²² For each of the 60 samples, 549 SNPs were analysed on chromosome 13 and 570 SNPs were analysed on chromosome 21. Chromosome 21 was selected for analysis because it is commonly associated with fetal abnormalities, whereas chromosome 13 was chosen because it is less commonly associated with fetal abnormalities, and thus could also serve as a reference chromosome.

SNPs were amplified from genomic DNA isolated from the maternal plasma, the maternal buffy coat, and

the paternal buffy coat by PCR. For all SNPs, the first primer contained a recognition site for the restriction enzyme *EcoRI* and had a biotin tag at the 5' end. The second primer contained a recognition site for a type IIS restriction enzyme.²² 38 cycles of PCR were done as follows: (1) 95°C for 15 min, (2) 94°C for 30 s, (3) 37°C for 30 s, (4) 94°C for 30 s, (5) 52°C for 30 s, (6) 94°C for 30 s, (7) 58°C for 30 s, (8) repeat steps 6 and 7 for 37 cycles, and (9) 72°C for 3 min.²² PCR products were bound to streptavidin-coated well plates for 1 h at 37°C with constant mixing at 400 rev per min with a Thermomixer (Eppendorf, Westbury, NY, USA).

After binding, all wells were washed three times with phosphate-buffered saline.

Bound PCR products were digested for 1 h at 55°C with the appropriate type IIS restriction enzyme to yield bound DNA fragments with a specific 5' overhang containing the SNP of interest, and a 3' recessed end. Wells were rinsed three times with phosphate-buffered saline. The bound DNA fragments were labelled at 55°C for 1 h with a mixture of fluorescently labelled dideoxynucleotide triphosphates, non-fluorescently labelled dideoxynucleotide triphosphates, and a DNA polymerase. Labelled PCR products were released from the wells by digestion with *EcoRI* for 1 h at 37°C with constant mixing at 400 rev per min.

3 µL of the resulting digests from each well were loaded onto sequencing gels. Gels were run for 1 h at 65 W constant power. Fluorescently labelled fragments were visualised with Typhoon 8600 and Typhoon 9400 variable mode imagers (Amersham/GE Biosciences, Piscataway, NJ, USA). Maternal and paternal DNA samples isolated from the corresponding buffy coats were loaded next to the three corresponding plasma DNA replicates. Bands representing each allele in the plasma sample (upper or lower), were delimited and the pixel density of each band was quantified with ImageQuant version 5.2 (Amersham/GE Biosciences, Piscataway, NJ, USA). Pixel density measurements were used to calculate a ratio of unique fetal allele signal to the combined maternal and fetal allele signal.

Figure 2 shows examples of category one SNPs on chromosomes 13 and 21 at different percentages of fetal DNA in the maternal circulation, showing allele signals from maternal buffy coat DNA, paternal buffy coat DNA, and maternal plasma DNA. The unique fetal DNA signal in the plasma matches the paternal allele, and is opposite the maternal allele. The ratio of the unique fetal allele signal to the combined signal from the maternal and fetal alleles was calculated at multiple SNP sites on chromosomes 13 and 21. Figure 3 illustrates how ratios at individual SNP sites on chromosome 13 and chromosome 21 were used to calculate the ratio of fetal to maternal DNA in the maternal plasma. This example is based on the quantification of a sample with trisomy 21 containing 50% fetal DNA in the maternal plasma.

Figure 3A shows the quantification of a SNP on chromosome 13 in a sample with trisomy 21. In this example, the fetus inherits one maternal and one paternal copy of chromosome 13. Therefore, the ratio of unique fetal allele signal to the combined maternal and fetal allele signal in the plasma is 1 to 3. At all SNP sites on chromosome 13, the ratio would be expected to approach 1 to 3, and over multiple SNP sites, the mean ratio would be very close to 0.333. Figure 3B shows the quantification of a SNP on chromosome 21 in a sample with trisomy 21. Because the fetus inherits two maternal copies and one paternal copy of chromosome 21, the

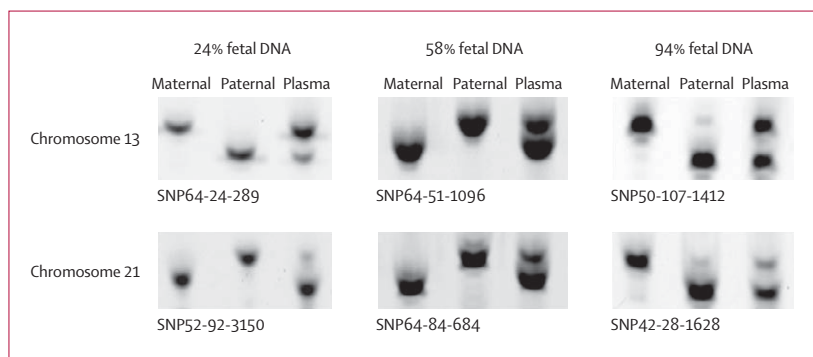


Figure 2: SNPs on chromosomes 13 and 21 at various fetal DNA percentages in maternal plasma samples
In a maternal plasma sample, the inherited paternal allele represents a unique marker, which distinguishes fetal DNA from maternal DNA. A homozygous allele signal from maternal genomic DNA (maternal), a homozygous allele signal from paternal genomic DNA (paternal) and signals from heterozygous alleles in maternal plasma DNA (plasma) are shown. The 94% fetal DNA example is sample 4, which was identified as trisomy 21. By contrast with the roughly 1 to 1 ratio of maternal (upper) and unique fetal (lower) alleles in the chromosome 13 panel (top), an indication of the presence of an additional, paternally inherited allele, is visible in the chromosome 21 panel (bottom), in which the ratio of maternal to unique fetal alleles is greater than 1 to 1.

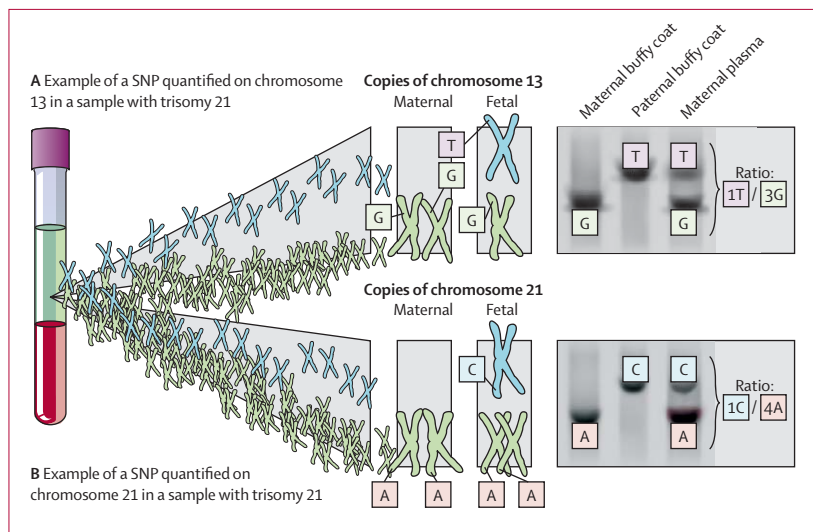


Figure 3: Establishing the copy number of fetal chromosomes through analysis of allele ratios in the maternal plasma

(A) Example of a SNP quantified on chromosome 13 in a sample with trisomy 21; the ratio of unique fetal signal ("T") to the combined maternal and fetal signal ("G") in the plasma is 1 to 3 and over many SNPs, approaches a theoretical mean ratio of 0.333. (B) Example of a SNP quantified on chromosome 21 in a sample with trisomy 21; the ratio of unique fetal signal to combined maternal and fetal signal is reduced to 1 to 4 and over many SNPs, approaches a theoretical mean ratio of 0.25; consequently, the mean ratio for chromosome 21 will be substantially lower than the mean ratio for chromosome 13.

ratio of unique fetal allele signal to the combined maternal and fetal allele signal would be expected to be 1 to 4 (ie, 0·25). A comparison of mean fetal DNA ratios for the two chromosomes would show that the mean ratio for chromosome 21 is significantly lower than the mean ratio for chromosome 13, thus indicating trisomy 21.

The fetal DNA ratio (R) was used to calculate the percentage (P) of fetal DNA in the plasma with the formula: $P = (2R/[1+R]) \times 100\%$. The mean log ratio for all SNPs on chromosome 13 and the mean log ratio for all SNPs on chromosome 21 from the three independent plasma replicates were calculated to test for differences between the two chromosomes.^{22,23}

Statistical analysis

All category one and two SNPs that were identified in the plasma DNA were quantified, meaning that for every sample, a different number of SNPs were quantified per chromosome. The natural log of the raw ratios was calculated and averaged across replicates, and then averaged across SNPs for each chromosome. The mean log ratio of fetal DNA between chromosomes 13 and 21 was compared by a two-tailed Student's *t* test allowing for unequal variances. The difference between the ratio of chromosome 13 and chromosome 21 fetal DNA was deemed to be significant when the significance level of the *t* test was less than 0·05. This process was repeated separately for data from all patient samples. All calculations were done with S-Plus version 7.0.

Role of the funding source

Ravgen, Inc designed and conducted the study; collected, managed, analysed, and interpreted the data; and prepared, reviewed, and submitted the manuscript. M Damewood had access to all the data in this study and takes responsibility for the integrity of the data and the integrity of the data analysis, and is not an employee of Ravgen, Inc. The corresponding author had final responsibility for the decision to submit the manuscript for publication.

Results

The median maternal age was 34 years; the median gestational age was 17 weeks and 5 days (table 1). The earliest gestational age at which the test was done was just over 8 weeks (sample 46; table 1). Eight of the samples were drawn in the first trimester.

The mean proportion of free fetal DNA was 34·0% (median 32·5%, range 17·0–93·8; table 2). 51 of 60 samples had more than 25% free fetal DNA. Of these samples, three had 50% or more free fetal DNA.

Table 2 shows the number of SNPs analysed on each chromosome for all samples. All category one and two SNPs identified in the plasma DNA were quantified. The number of SNPs analysed in the plasma fraction varied by sample, but a mean of 22 SNPs were analysed on chromosome 13, and 20 SNPs were analysed on

	Maternal age (years)	Gestational age (weeks and days)
1	36	16 weeks 0 days
2	27	26 weeks 6 days
3	34	18 weeks 1 day
4	43	20 weeks 1 day
5	20	18 weeks 5 days
6	41	16 weeks 4 days
7	30	19 weeks 1 day
8	37	17 weeks 1 day
9	35	16 weeks 3 days
10	29	17 weeks 0 days
11	37	16 weeks 6 days
12	37	19 weeks 1 day
13	39	17 weeks 2 days
14	27	17 weeks 4 days
15	34	17 weeks 3 days
16	40	18 weeks 1 day
17	41	17 weeks 5 days
18	36	17 weeks 5 days
19	35	18 weeks 1 day
20	31	21 weeks 2 days
21	33	15 weeks 6 days
22	29	20 weeks 0 days
23	25	36 weeks 5 days
24	35	17 weeks 0 days
25	22	14 weeks 1 day
26	34	22 weeks 4 days
27	36	35 weeks 6 days
28	38	16 weeks 6 days
29	30	15 weeks 6 days
30	30	11 weeks 4 days
31	36	19 weeks 2 days
32	37	17 weeks 4 days
33	31	16 weeks 3 days
34	34	29 weeks 5 days
35	42	11 weeks 6 days
36	24	13 weeks 6 days
37	32	16 weeks 1 day
38	31	32 weeks 1 day
39	35	18 weeks 6 days
40	38	12 weeks 1 day
41	34	20 weeks 0 day
42	34	21 weeks 2 days
43	30	12 weeks 2 days
44	32	37 weeks 2 days
45	27	26 weeks 1 day
46	30	8 weeks 1 day
47	33	17 weeks 0 days
48	34	27 weeks 5 days
49	34	11 weeks 1 day
50	33	15 weeks 1 day
51	34	18 weeks 3 days
52	18	35 weeks 6 days

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chromosome 21. Significant differences in the ratio of fetal to maternal DNA were seen in samples 4 ($p=0.04$), 18 ($p=0.05$), and 31 ($p=0.04$).

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53	31	16 weeks 5 days
54	21	28 weeks 0 days
55	33	16 weeks 4 days
56	43	36 weeks 6 days
57	31	38 weeks 6 days
58	34	10 weeks 6 days
59	26	26 weeks 1 day
60	36	28 weeks 0 days
Median	34	17 weeks 5 days

Table 1: Maternal age and gestational age for the 60 patient samples

	Chromosome	Number of SNPs quantified	Ratio of fetal to maternal DNA	Difference in fetal DNA ratio (13 vs 21)	Percentage fetal DNA	p value*
1	13	13	0.3209	0.0960	48.6%	0.15
	21	11	0.2249			
2	13	40	0.4032	-0.0421	57.5%	0.43
	21	33	0.4453			
3	13	27	0.1959	-0.0260	32.8%	0.44
	21	29	0.2219			
4	13	46	0.8826	-0.3621	93.8%	0.04†
	21	35	1.2446			
5	13	18	0.1489	-0.0025	25.9%	0.95
	21	23	0.1514			
6	13	24	0.0928	0.0117	17.0%	0.45
	21	24	0.0811			
7	13	13	0.1468	-0.0060	25.6%	0.88
	21	21	0.1528			
8	13	16	0.1217	-0.0098	21.7%	0.75
	21	14	0.1315			
9	13	22	0.1528	0.0092	26.5%	0.74
	21	24	0.1436			
10	13	28	0.1486	-0.0433	25.9%	0.27
	21	17	0.1919			
11	13	18	0.2335	0.0604	37.9%	0.12
	21	23	0.1732			
12	13	12	0.1504	0.0217	26.2%	0.59
	21	8	0.1288			
13	13	41	0.1576	-0.0420	27.2%	0.16
	21	17	0.1996			
14	13	19	0.0994	-0.0099	18.1%	0.68
	21	17	0.1093			
15	13	17	0.1347	0.0196	23.7%	0.42
	21	30	0.1151			
16	13	7	0.2126	0.0528	35.1%	0.19
	21	15	0.1599			
17	13	25	0.1478	-0.0696	25.7%	0.10
	21	11	0.2174			

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Amniocentesis or newborn reports from the clinical sites confirmed that the copy number of fetal chromosomes 13 and 21 was determined correctly for 58 out of the 60 samples analysed; our method correctly identified 56 of the 57 normal samples, and two of the three trisomy 21 samples (samples 4 and 31). Sample 18 was a false positive ($p=0.05$) on the basis of a negative amniocentesis report for the sample. Sample 55, which was identified as trisomy 21 by amniocentesis was falsely identified as a normal sample by our methods (ie, was a false negative; $p=0.34$). The sensitivity of our test was 66.7% (95% CI 12.5–98.2), specificity was 98.2% (89.4–99.9), positive predictive value was 66.7% (12.5–98.2), and the negative predictive value was 98.2% (89.4–99.9). Of the 57 true normal samples, the median maternal age was 34 years and the median gestational age was 17 weeks and 4 days. Of the three true abnormal samples, the median maternal age was 36 years and the median gestational age was 20 weeks and 1 day (table 1).

In sample 4, the mean ratio of fetal DNA for chromosome 21 was significantly higher than that for chromosome 13, indicating trisomy 21 in the fetus, and that the additional copy was inherited from the paternal genome ($p=0.04$; table 2). In sample 31, the mean ratio of fetal DNA for chromosome 21 was significantly lower than that for chromosome 13, indicating trisomy 21 in the fetus, and that the additional copy was inherited from the maternal genome ($p=0.04$; table 2).

Discussion

By use of this method to detect and quantify fetal DNA in maternal plasma, the copy number of fetal chromosomes of interest—eg, chromosomes 13 and 21—can be determined from maternal blood samples, and does not require isolation of fetal cells or free fetal DNA. Venipunctures are done routinely in clinical settings and present little risk to the mother and fetus.

Many of the participants that we tested were from the patient population for which testing for aneuploidy is recommended; the mothers had a median age of 34 years. The median gestational age of the fetus (17 weeks and 5 days) is within the time-frame when invasive diagnostic procedures are commonly done. The earliest gestational age analysed in this study was just over 8 weeks, and resulted in the correct identification of a normal copy number of chromosomes 13 and 21. Of the eight first-trimester samples analysed, all were correctly identified as being normal. Thus, the methods described herein serve the appropriate patient population.

Amniocentesis or newborn reports from the clinical sites confirmed that the copy number of fetal chromosomes 13 and 21 was determined correctly for 58 out of the 60 samples analysed, including two of the three cases of trisomy 21. A probability calculation shows that the chances of identifying two or more of the three

cases of trisomy 21 in the 60 observations, if the test had zero diagnostic power, is 0·005.

At present, prenatal testing includes a combination of available diagnostic and screening tests. Screening tests include the first-trimester screen (ultrasound-based nuchal translucency measurement with maternal serum analyte biochemistry), second-trimester maternal triple or quadruple serum analyte biochemistry, and second-trimester fetal ultrasound. Screening tests for Down's syndrome are often reported to have a 5% false positive rate, with 64–96% of cases of trisomy 21 being identified.^{1,24,25} The goal of any screening test is to increase the number of diagnoses made to a maximum while reducing the number of false positives to a minimum. Such a goal would decrease the number of follow-up invasive diagnostic tests—eg, amniocentesis or chorionic villus sampling.

A practice bulletin from the American College of Obstetricians and Gynecologists, published in January, 2007, recommends that all women should be offered the option of genetic screening, irrespective of maternal age.²⁵ Assuming that a larger number of women will be tested, and given the 5% false positive rate associated with available screening methods, this recommendation will probably increase the need for invasive diagnostic tests that are associated with a higher risk of miscarriage. In turn, it follows that there will be a greater demand for the development of non-invasive tests that yield diagnostic results.

To achieve a false positive rate of 1%, the detection rate of available screening tests drops to a range of 45–88%.¹ On the basis of our results, the SNP ratio method described here achieves a false positive rate under 2% with a detection rate of 66%. Since we report only 60 observations, including three cases of trisomy 21, estimates of the sensitivity, specificity, and positive and negative predictive values of the test are preliminary. Larger trials are needed to confirm that test performance is reproducible and comparable to available tests. Refinements to the method—eg, quantifying a greater number of SNPs and increasing the number of reference chromosomes—should enhance test performance.

One of the samples identified as a case of trisomy 21 by amniocentesis was falsely identified as a normal sample using our methods (sample 55, $p=0\cdot34$). Additionally, one of the samples identified by our methods as a trisomy 21 sample was a false positive (sample 18, $p=0\cdot05$). Large-scale clinical trials are needed to more accurately establish the specificity and sensitivity of the test, and to determine the optimum number of SNPs needed to identify chromosomal abnormalities at a range of free fetal DNA concentrations.

There is generally less variation in mean fetal DNA ratios between chromosomes as more SNPs are quantified. For instance, sample 4, which was identified

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18	13	11	0·1218	–0·0772	21·7%	0·05†
	21	10	0·1990			
19	13	17	0·1828	0·0121	30·9%	0·78
	21	19	0·1707			
20	13	12	0·1712	0·0291	29·2%	0·29
	21	16	0·1421			
21	13	31	0·1759	–0·0088	29·9%	0·81
	21	19	0·1847			
22	13	27	0·1504	0·0108	26·1%	0·67
	21	36	0·1396			
23	13	37	0·3465	0·0517	51·5%	0·27
	21	32	0·2948			
24	13	27	0·1932	–0·0256	32·4%	0·58
	21	22	0·2188			
25	13	20	0·1351	–0·0148	23·8%	0·70
	21	13	0·1499			
26	13	23	0·1947	0·0438	32·6%	0·24
	21	19	0·1509			
27	13	26	0·2286	–0·0616	37·2%	0·25
	21	19	0·2902			
28	13	9	0·1532	–0·0845	26·6%	0·16
	21	17	0·2377			
29	13	28	0·1835	0·0026	31·0%	0·94
	21	14	0·1809			
30	13	27	0·1938	0·0350	32·5%	0·31
	21	20	0·1588			
31	13	34	0·2704	0·0877	42·6%	0·04†
	21	23	0·1827			
32	13	19	0·1674	–0·0450	28·7%	0·43
	21	9	0·2125			
33	13	17	0·2050	–0·0172	34·0%	0·78
	21	9	0·2223			
34	13	25	0·1840	–0·0229	31·1%	0·70
	21	15	0·2070			
35	13	13	0·2043	–0·0179	33·9%	0·73
	21	13	0·2221			
36	13	20	0·3329	–0·0217	49·9%	0·74
	21	18	0·3546			
37	13	27	0·2980	–0·0409	45·9%	0·56
	21	17	0·3389			
38	13	16	0·2765	0·0204	43·3%	0·76
	21	26	0·2561			
39	13	9	0·3320	0·0940	49·9%	0·13
	21	26	0·2380			
40	13	31	0·2755	–0·0174	43·2%	0·76
	21	28	0·2929			
41	13	17	0·1601	0·0306	27·6%	0·35
	21	18	0·1295			
42	13	15	0·1219	–0·0056	21·7%	0·88
	21	12	0·1275			
43	13	27	0·1378	0·0424	24·2%	0·16
	21	13	0·0954			
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Articles

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44	13	21	0.2638	0.0127	41.8%	0.76
	21	20	0.2511			
45	13	22	0.1808	-0.0215	30.6%	0.56
	21	15	0.2023			
46	13	16	0.1367	0.0110	24.1%	0.73
	21	17	0.1257			
47	13	17	0.2450	-0.0751	39.4%	0.27
	21	15	0.3201			
48	13	29	0.1943	0.0055	32.5%	0.89
	21	20	0.1887			
49	13	20	0.1615	-0.0313	27.8%	0.39
	21	20	0.1928			
50	13	16	0.2307	0.0491	37.5%	0.32
	21	18	0.1816			
51	13	19	0.2617	0.0427	41.5%	0.44
	21	14	0.2189			
52	13	18	0.2079	-0.0696	34.4%	0.24
	21	19	0.2775			
53	13	25	0.1570	0.0279	27.1%	0.27
	21	29	0.1291			
54	13	21	0.2023	0.0261	33.6%	0.61
	21	13	0.1762			
55	13	25	0.1887	-0.0310	31.8%	0.34
	21	23	0.2197			
56	13	20	0.2053	-0.0022	34.1%	0.94
	21	43	0.2075			
57	13	30	0.2661	0.0238	42.0%	0.58
	21	38	0.2423			
58	13	21	0.2338	0.0077	37.9%	0.88
	21	26	0.2261			
59	13	30	0.1978	0.0520	33.0%	0.11
	21	18	0.1458			
60	13	37	0.2723	-0.0150	42.8%	0.75
	21	29	0.2873			

*Compares mean fetal DNA ratios for chromosome 13 and chromosome 21. †Indicates significant difference between fetal DNA ratios for chromosome 13 and chromosome 21 ($p < 0.05$).

Table 2: Comparison of mean fetal DNA ratios for chromosome 13 and 21 in 60 maternal plasma samples

correctly as a case of trisomy 21, had the highest percentage of free fetal DNA in the study (93.8%), and a large number of SNPs were detected in the plasma of this sample (46 SNPs on chromosome 13 and 35 SNPs on chromosome 21), which facilitated the identification of trisomy 21. By contrast, the false positive result (sample 18) had the second lowest percentage of free fetal DNA and the second lowest total number of SNPs analysed (11 on chromosome 13 and ten on chromosome 21), which raises the possibility of false positive results occurring when too few SNPs are analysed in a sample with a low percentage of free fetal DNA. Therefore, to define the optimum number of SNPs that provide highly accurate diagnostic information and that can be analysed in an efficient manner is essential.

Quantification of fewer SNPs could be sufficient at high percentages of free fetal DNA and more SNPs can be analysed when the percentage of free fetal DNA is low. To establish the optimum number of SNPs needed for diagnosis of trisomy 21 and other clinically relevant trisomies is important. Given that eight first-trimester samples, including a sample drawn at about 8 weeks' gestation, were correctly identified, and that phlebotomy is minimally invasive, there exists the possibility of analysing follow-up samples to identify a greater number of SNPs of the correct pattern, should a low number of SNPs of the correct pattern be identified in the initial sample.

In this study, paternal genomic DNA was genotyped to reduce the number of SNPs analysed in the plasma sample. Inclusion of paternal DNA was strictly for reference purposes and is not required for quantitative analysis of fetal DNA. Quantitative analysis is achieved through a comparison of maternal buffy coat DNA and maternal plasma DNA from a single sample of the mother's blood.

Importantly, the number of reference chromosomes that can be studied with this method is not limited. We compared chromosome 13 with chromosome 21, but any number of chromosomes can be compared, including chromosomes 18, X, and Y, together with reference chromosomes (eg, chromosomes 15 and 22). The scope and accuracy of this method would likely be increased by comparing a larger number of chromosomes and a larger number of SNPs on each chromosome. Furthermore, the human genome project has identified over 3.7 million SNPs to date. Even on the smallest human chromosome—chromosome 21—about 54 000 genotyped SNPs are available for analysis. Clearly, there are additional SNPs that could be added to the current test.

The approach described here, which uses standard molecular biology equipment, allows for precise analysis of genetic material. Free fetal DNA is directly quantified from the heterogeneous mixture of maternal and fetal DNA in the maternal plasma. The maternal signal in the plasma DNA serves as an internal control, thus reducing variability. Rather than calculating absolute readings of the fetal-specific alleles, the ratio of unique fetal-specific allele signals to the combined maternal and fetal allele signal is determined. Since the difference between the mean ratios for chromosome 13 and 21 is directly compared, the effect of systematic variations originating from experimental processing and variations in SNPs in the genome can be kept to a minimum.

Our results show that SNPs can be used to distinguish fetal DNA from maternal DNA—and to determine the copy number of fetal chromosomes—in maternal blood samples. With further refinement, a prenatal diagnostic test based on the methods described here could be a useful complement to currently available prenatal tests.

Contributors

R Dhallan and P Bayliss conceived and designed the study. X Guo, S Emche, J Betz, J Barry, K Franz, K Gold, B Vallecillo, and J Varney took part in the acquisition of data. R Dhallan, X Guo, S Emche, M Damewood, P Bayliss, and M Cronin analysed and interpreted the data. R Dhallan, X Guo, S Emche, M Damewood, P Bayliss, M Cronin, J Barry, J Betz, K Franz, K Gold, B Vallecillo, and J Varney drafted the manuscript. R Dhallan, M Damewood, P Bayliss, and M Cronin critically reviewed the manuscript. R Dhallan, X Guo, and M Damewood provided statistical expertise. R Dhallan obtained funding and supervised the study. R Dhallan, X Guo, S Emche, M Damewood, P Bayliss, M Cronin, J Barry, J Betz, K Franz, K Gold, B Vallecillo, and J Varney provided administrative, technical, or material support.

Conflict of interest statement

R Dhallan is the founder, chief executive officer, and chairman of the board of directors of Ravgen, Inc, and a stockholder in Ravgen, Inc. M Cronin is a stockholder in Ravgen, Inc. S Emche, X Guo, J Barry, J Betz, K Gold, B Vallecillo, and J Varney are employed by, and have options to purchase stock in, Ravgen, Inc. K Franz was employed by Ravgen at the time of the study and has options to purchase stock in Ravgen, Inc. P Bayliss is a member of the board of directors of Ravgen, Inc, and has options to purchase stock in Ravgen, Inc. Marian Damewood is an unpaid member of Ravgen's advisory board, and has options to purchase stock in Ravgen, Inc. Ravgen, Inc has been issued a patent and has multiple patent applications pending for the methods described in this paper.

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